Biomatter 2:1, 15–23; January/February/March 2012; © 2012 Landes Bioscience

Resolving the CaP-bone interface

A review of discoveries with light and electron microscopy

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Keywords: calcium phosphate, electron microscopy, light microscopy, interface, bioceramic, focused ion beam, bone, hydroxyapatite

Abbreviations: AFM, atomic force microscopy; BFTEM, bright-field transmission electron microscopy; Cryo-FIBSEM, cryogenic focused ion beam scanning electron microscopy; Cryo-TEM, cryogenic transmission electron microscopy; CT, computed tomography; DFTEM, dark-field transmission electron microscopy; EDS, energy dispersive X-ray spectroscopy; EELS, electron energy loss spectroscopy; EFTEM, energy-filtered transmission electron microscopy; EM, electron microscopy; FIB, focused ion beam; HA, hydroxyapatite; HAADF, high-angle annular dark-field; LM, light microscopy; OCP, octacalcium phosphate; SEM, scanning electron microscopy; STEM, scanning transmission electron microscopy; TEM, transmission electron microscopy

It has long been known that the interfacial relationship between synthetic materials and tissue is influential in the success of implant materials. Instability at the implant interface has been shown, in some cases, to lead to complete implant failure. Bioceramics, and in particular calcium phosphates, form a large fraction of the implantable devices on the market today due to the biocompatibility they exhibit in contact with bone and tooth-like tissues. The characterization of such bioceramic-tissue interfaces has played a crucial role in understanding the behavior of bioceramics in vivo. In this review, we shed light on the preparation methods, technological approaches and key advances in resolving the interface between calcium phosphate bioceramics and bone, and share a future outlook on this field.

Introduction

One of the most thoroughly investigated categories of bioceramics is calcium phosphates. This broad term envelops materials such as hydroxyapatite (HA), tricalcium phosphates, tetracalcium phosphates, octacalcium phosphates (OCP) and ion-substituted forms of the aforementioned. Calcium phosphate ceramics have vast applications in the biomedical field due to their similar composition to the mineral component of bone. Bone mineral is believed to be a combination of HA and ion-substituted apatites.[1-4] The calcium phosphate interface in vivo is dynamic, particularly at the biological side, thereby allowing bone formation both into and from the CaP surface.[5,6]

Calcium phosphates for bone regrowth and regeneration are available in a number of forms including granules, blocks, injectable cements, scaffolds and coatings.[3,7] Of the properties associated with these bioceramics, biocompatibility is perhaps one of the most important factors to consider. Biocompatibility was traditionally defined as the ability of a material to elicit an appropriate host response.[8,9] In the case of implants intended for contact with hard tissue, such as bone, the appropriate host response involves osseointegration; the direct contact between living bone and implant surface.[10] Originally, when this term was defined, the requirement for contact was on the light microscopic resolution level. However, electron microscopy enables evaluation of materials at a much higher resolution level and allows determination of direct contact on a nanometer or ultrastructural level. As a result, we have seen an evolution in the understanding of the bioceramic-bone interface in accordance with the progression of characterization techniques, from light optical microscopy (LM), to scanning electron microscopy (SEM), to transmission electron microscopy (TEM) and focused ion beam microscopy (FIB). Herein, we focus mainly on the role of light and electron microscopy in the comprehension of the calcium phosphate bioceramic interface to bone in vivo.

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Submitted: 11/18/11; Revised: 01/20/12; Accepted: 03/09/12
http://dx.doi.org/10.4161/biom.20062

Sample Preparation Approaches

The greatest challenge in resolving the interface between biomaterials and bone remains sample preparation. Methods to prepare samples vary widely depending on the requirements of the characterization technique. Indeed, as we move from LM to SEM to TEM the complexity of the technique, and therefore the associated sample preparation method, increases.

In all cases, the first challenge is removing an intact sample from the in vivo environment. Depending on implant geometry and location, standard removal approaches include trephine drill or low speed saw. Implementing precautionary measures, to reduce heat and mechanical forces, by the use of low-speed instruments and flushing with cool aqueous solutions can aid in the removal of intact tissue-implant specimens. As mentioned, the subsequent sample preparation procedures vary depending on the characterization technique that follows.

Tissue processing. Most samples investigated by light and SEM require embedding in resin or paraffin wax. The standard biological tissue processing method involves the sequential fixation in glutaraldehyde or formaldehyde, post-fixation in osmium tetroxide, dehydration in ethanol or acetone, followed by embedding in epoxy or acrylic based resins including PMMA or paraffin.[11-13] The choice of embedding medium may be an important factor to consider since it may influence the degree of tissue-implant interfacial separation. For instance, the use of Technovit and Epon as embedding media have shown significantly less separation than LR White.[14] Similarly, the choice of embedding media also determines the thickness of sections that can be prepared for subsequent LM or EM investigations. Due to the softness of paraffin compared with plastic resins, sections of comparable thinness are not achievable.[15] Additionally, paraffin embedding often requires decalcification and recent investigations have shown that calcified bone embedded in Technovit provides superior preservation of trabecular bone structure and yields stronger immunostaining.[16]

Sectioning, grinding and staining. In their article on the Säge-Schliff (sawing and grinding) technique, Donath and Breuner have outlined a method for the preparation and sectioning of implant materials in contact with bone for histological and morphological evaluation by LM.[17] Using this procedure or variations of it, the resultant embedded bone-implant specimens are 5–10 μm thick with the possibility of applying stains or immunogold labeling.[18,19] In LM and SEM, staining of bone samples, with for example toluidine blue, Alizarin red, lead citrate or other heavy metals, is common practice for evaluating the stages of bone growth or improving contrast.[20-22] Furthermore, immunogold labeling plays a key role in detecting the presence of bone specific matrix proteins such as fibronectin and osteonectin at an implant interface. Preparation of sections in this manner has enabled LM and SEM of the interface between calcified tissues and implant, allowing histological and morphometric evaluation of the short- and long-term tissue response to implanted materials.

Ultramicrotomy. To proceed with investigations of the tissue-material interface on the nanometer range, it is essential that samples are electron transparent for TEM studies. In the case of biomaterials, electron transparency corresponds to a minimum thickness between 50–100 nm.[23] Achieving such specimens has traditionally been done by ultramicrotomy, the slicing of thin samples with a diamond blade followed by their collection onto a TEM grid. Maintaining the contact between soft biological tissues and brittle ceramics, while maintaining the integrity of biological shapes, is often a challenge with this technique. As a result, ion milling techniques have become the preferred choice for TEM sample preparation of calcium phosphates interfacing to bone.

Ion milling. One alternative technique to ultramicrotomy for producing ultrathin electron transparent specimens is broad beam ion milling. The comparison between broad beam ion milling and FIB milling, discussed below, has shown little difference in the quality of resultant specimens.[24] Similarly to ultramicrotomy, the drawback of broad beam ion milling is the inability to select the exact interface region of interest, and therefore there is an associated risk that not all specimens produced will contain the region of interest within them.

Focused ion beam (FIB). FIB instruments have been used widely in the materials science and microelectronics industry.[25] However, the use of dual beam FIB and SEM is gaining popularity in the life sciences. For the preparation of biomaterial-bone interfaces, the FIB presents several advantages to conventional preparation using ultramicrotomy or broad beam ion techniques. First, the coupling of the ion beam with a SEM instrument enables the site-specific selection of interfaces for study by TEM. In addition, the ion beam current or energy can be reduced sufficiently to maintain intact interfaces throughout preparation. This is particularly a challenge in ultramicrotomy, where materials of considerably different hardness values often separate under the cutting blade.

FIB sample preparation has been successfully demonstrated for surface analysis of implant materials,[26,27] the interfaces between titanium and bone,[28,29] titanium with magnetron sputtered HA and bone,[30] cells and HA,[31] HA and bone,[32] glass ionomer cements and dentin,[24] calcium aluminate coatings and bone[14] and pulsed-laser deposited HA on titanium,[33] to name a few. In all these examples, the FIB instrument produced samples of quality equivalent or greater than that of broad beam, tripod polished or ultramicrotomed samples, and did so while maintaining contact between materials of varying hardness.

Nevertheless, it is important to note that there are a number of artifacts associated with the use of FIB instruments.[34-36] Although precautions should be taken to avoid ion beam-induced damage, it is possible that a small amount of gallium ion implantation may occur, which is problematic if quantitative chemical analysis by TEM methods is of concern. Moreover, differential sputtering rates and ion channeling may result in a 'theater curtain' effect across the sample, consisting of vertical striped regions corresponding to different milling rates, which may distort features of interest.[34] Lastly, redeposition of sputtered material can occur on the sample surface if the beam dose is too high, not accurately placed or if the beam is not finely tuned.[34] Experience, a carefully tuned instrument and, more recently, the use of low energy ion cleaning steps may help reduce the likelihood of artifact occurrences.[37,38]

Although not used for the study of bioceramics, Giannuzzi et al. have demonstrated the ability to use FIB sequential milling and imaging to produce three dimensional reconstructions of biomaterial-bone interfaces.[39] Such three dimensional interfacial information was useful for determining the extent of bone ingrowth into implanted titanium samples, and could easily be applied to the bioceramic interface in much the same way.

Cryo-preparation. The use of cryogenic-based techniques is quite common in the study of biological tissues. Cryofracturing has been used by Steflik et al. to ensure interfacial tissues remain intact on tissue blocks when removing implants from apposing tissue.[40] Of late, advancements in cryo-FIBSEM and cryo-TEM instrumentation have enabled the complete preparation, transfer and investigation of biological specimens from start to finish under cryogenic conditions, eliminating the need for rigorous

tissue processing.[41] This may in fact be the best route for preparing and examining biological structures in their native state.

Resolving the Interface: The Techniques

Light microscopy (LM). Ground sections must be produced, as described earlier, for light microscopic evaluation of the intact interface between non-demineralized tissue and implants, so that features such as bone-implant contact and bone area can be quantified. It has been shown that the thickness of the ground sections are of importance for quantification, as thicker samples include more overlapping information and often result in an overestimation of bone implant contact.[42] Furthermore, the cutting direction may influence the quality of sections, thereby also interfering with quantification.[43] Different staining protocols enable the identification of features for qualitative histology, such as discrimination of woven bone tissue, mature bone tissue, as well as cellular activity. Additionally, with the injection of calcium binding dyes during healing, the mineralization front can be tracked in the ground-section, contributing information regarding, e.g., the origin of bone tissue growth. Linear or circular polarized light microscopy, covered comprehensively elsewhere,[44] may also be employed to identify the orientation of collagen in bone, enabling identification of regions of woven or lamellar bone in contact with calcium phosphates.[45]

Scanning electron microscopy (SEM). The scanning electron microscope is a valuable characterization tool for bone-implant interfacial analysis. Of the variety of electron-matter interactions that occur in the SEM (**Fig. 1**), the detection of backscattered electrons is the most useful in the study of calcium phosphates and bone. Backscattered electrons are highly Z-dependent and therefore create Z-contrast images. This is crucial for studying calcium phosphates in contact with bone, since their chemical similarities make them difficult to distinguish otherwise. The gold standard in determining percentage of bone-implant contact and bone-in-growth area is backscattered SEM.[46] In addition to its application for bone contact measurements, backscattered electrons are useful for choosing sites of good bone-implant contact for further investigation with TEM. SEM can also be used to obtain analytical information from the sample by collecting the characteristic X-rays emitted with energy dispersive X-ray spectroscopy.

Transmission electron microscopy (TEM). In the TEM, electrons are transmitted through an extremely thin (≤ 100 nm) sample. A variety of signals can be detected (**Fig. 1**) and therefore the TEM has a number of different techniques that are useful for the analysis of bioceramic-bone interfaces. Imaging can be achieved using bright or dark-field TEM (BFTEM, DFTEM) or high-angle annular dark-field (HAADF) in scanning transmission electron microscopy (STEM). Since the HAADF detector collects incoherently scattered electrons, the image is highly Z-dependent and thus images with compositional contrast are attainable.

Elemental analysis of samples can be achieved via many routes in the TEM including energy dispersive X-ray spectroscopy (EDS), energy filtered TEM (EFTEM) or electron energy loss spectroscopy (EELS). Gregori et al. demonstrated that EELS is an effective method to differentiate between individual grains of HA and β-tricalcium phosphate by analyzing the oxygen K ionization edge.[47]

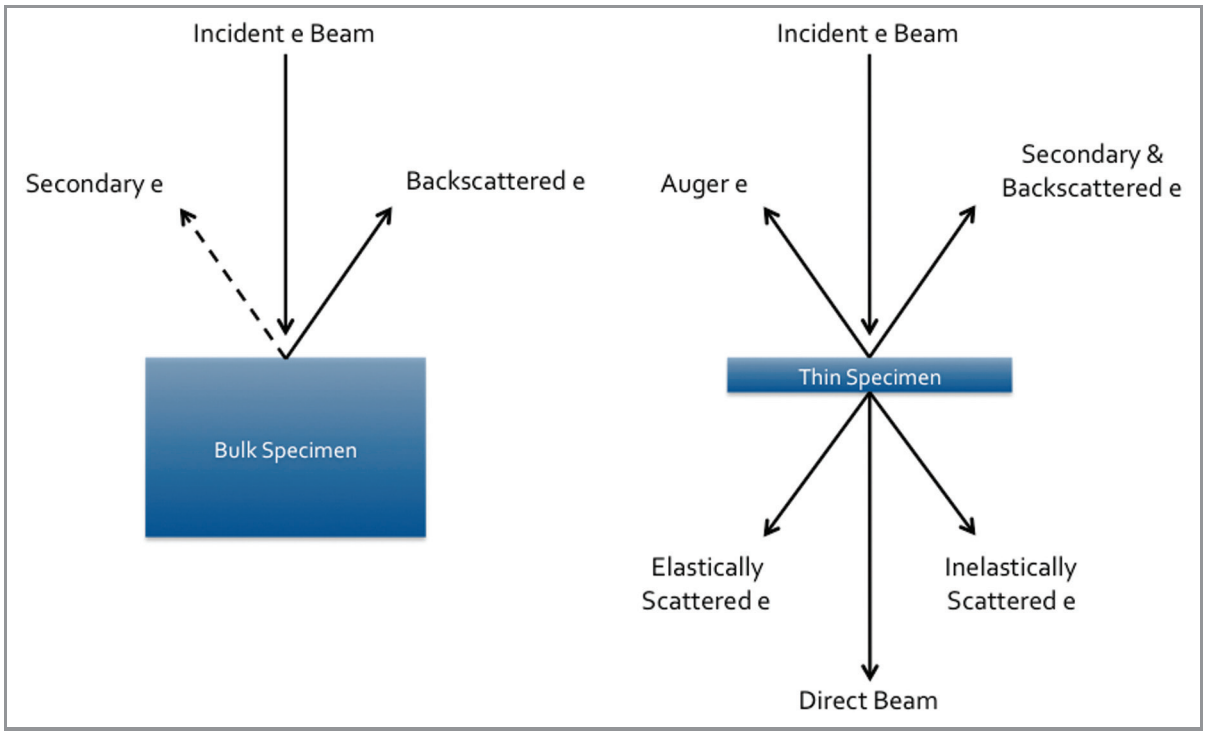

Figure 1. Signals generated in the SEM and TEM from electron-specimen interaction.

Atomic force microscopy (AFM). Scanning probe microscopies rely on the interaction between a physical probe and the sample surface and indeed have been applied to the study of various biomaterial interfaces. AFM is capable of producing high-resolution images, comparable to that of TEM, without the requirement of electron transparency. Since the technique is extremely surface sensitive, sample preparation of interfaces remains a challenge. For example, Yoshida et al. demonstrated the success of diamond knife cutting vs. polishing in their study of the dentin-resin interface with AFM.[48] Other studies have shown the potential of AFM as a tool to study biomechanical properties, such as Young's Modulus, at the interface between titanium and bone.[49] AFM has played an influential role in understanding mechanisms at the bioactive glass surface in vitro, for example, tracking the growth, distribution and roughness of globular like deposits formed in serum-free and serum-containing solutions.[50] AFM remains a relatively unexplored instrument in the study of bioceramic-bone interfaces. However, permitting the proper surface conditions, AFM offers a high-resolution alternative to TEM.

Resolving the HA-Bone Interface: Discoveries with LM and EM

LM fluorochrome labeling has indicated that bone formation away from the implant surface is 30% faster than formation of bone toward the implant, which signifies the importance of understanding the HA surface in vivo.[51] A variety of competing theories regarding the mechanisms of bone growth, the effect of properties such as crystallinity and porosity and the presence of an interfacial apatite layer, exist. These are discussed further.

Mechanisms of bone growth at the HA surface. It is well known that HA is a bioactive material, in that it precipitates an apatite layer on its surface in vivo, enabling it to form a chemical bond with bone.[2] Such bone bonding capabilities are of particular interest in the bone regeneration field. The proposed mechanism for bone bonding is a dissolution-reprecipitation process to form a biologically active apatite layer on the HA surface.[5,52-54] Ducheyne et al. have outlined the possible processes occurring on the surface of an HA sample in vivo. Eleven interactions occur simultaneously on the implant surface: (1) dissolution from the ceramic; (2) precipitation from solution onto the ceramic; (3) ion exchange and structural rearrangement at the ceramic-tissue interface; (4) interdiffusion from the surface boundary layer into the ceramic; (5) solution-mediated effects on cellular activity; (6) deposition of either (a) the mineral phase or (b) the organic phase, without integration into the ceramic surface; (7) deposition with integration into the ceramic; (8) chemotaxis to the ceramic surface; (9) cell attachment and proliferation; (10) cell differentiation; and (11) extracellular matrix formation.[52] Since the in vivo situation, as demonstrated, is extremely complex, many simplifications have been drawn by studying HA in vitro. In simulated body fluid solution, the formation of surface apatite is easily explained by the recruitment of calcium and phosphorus ions from the medium to the implant material due to the surface charge, forming an amorphous calcium phosphate layer illustrated in **Figure 2**.[55] Furthermore, recent advanced cryo-TEM and cryo-electron tomography techniques have uncovered the sequence of biomineralization in vitro, suggesting that the formation of pre-nucleation clusters proceeds the formation of amorphous apatite.[56] From these studies, we gain insight into processes that could occur in an in vivo environment.

Effect of materials properties. If we accept that initial bone growth is governed by a dissolution-reprecipitation method, then it is important to understand the dissolution behavior of calcium phosphates in vivo. A number of factors influence the rate of dissolution such as composition, crystallinity, grain size/boundaries and porosity.

Crystallinity. Upon dissolution in vivo, the interface takes on a jagged, rough surface exhibiting increased dissolution at grain boundaries (**Fig. 3A**).[57,58] In addition, reduced crystallinity has been noted in the vicinity of triple point grain boundary junctions

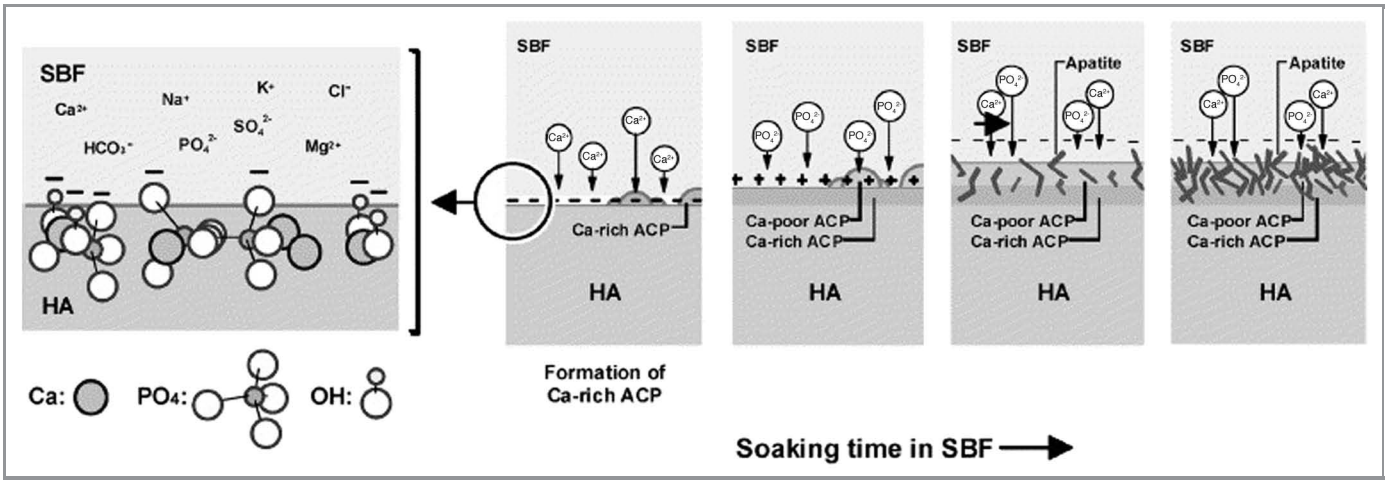

Figure 2. Schematic diagram representing the formation of amorphous calcium phosphate apatite on the surface of HA in vitro. Reproduced with permission from reference 55.

(**Fig. 3B**).[58] Annealed and crystalline coatings of plasma-sprayed HA have been shown to take longer to produce crystallites on their surface vs. a less crystalline coating, likely due to the reduced solubility of more crystalline materials.[59] Daculsi et al. also showed that line defects, representing a disruption in crystallinity, acted as starting points for dissolution of both biological and synthetic apatites.[54]

Porosity. It is well accepted that a macroporous structure of 100–400 μm is required to promote bone ingrowth.[53] In addition, Jones et al. have identified a cut-off accessible pore radius of 100 μm for cellular activity required to promote bone growth.[60,61] However, the presence of micropores is of increasing importance in the development of bone ingrowth. With SEM, micropores of 5 μm are regularly noted in HA samples, and generally located at grain boundaries (**Fig. 4**). It has been shown that the presence of these micropores may aid in resorption or dissolution by macroscopic break up rather than phagocytosis.[53] With the aid of LM, it has been demonstrated that, in otherwise identical macroporous scaffolds, the presence of microporosity increases bone growth.[62]

Chemistry. The doping of HA with Si has been found to destabilize the HA crystal structure, and reduce grain size, thereby increasing dissolution rates which result in increased bone apposition.[41,58] Interestingly, other studies have indicated that the behavior of various tricalcium phosphates, tetracalcium phosphates and HA samples in vivo is irrespective of their calcium to phosphorous ratio.[63] Furthermore, it has been noted that the ceramic behavior in vivo is irrespective of implant site, species and implantation time.[64]

The interfacial layer. To date, a consensus on the exact behavior of calcium phosphates in vivo, and in particular HA, has not been reached in the scientific community. Competing views on the surface formation of an interfacial apatite layer, its composition and its orientation with respect to bioceramic implant, exist. The surface behavior of HA in various forms has been studied thoroughly. Light microscopic techniques have shown evidence of a dark non-collagenous interfacial zone (**Fig. 5**).[45] Similarly, many TEM studies have identified a collagen free layer,[65] ranging in thickness from 20–1000 nm.[52,65,66] The composition of this layer has been suggested to be the inorganic matrix of bone and non-collagenous proteins. Steflik et al. identified interdigitating canaliculi connecting the interfacial layer to osteocytes outside the layer, indicating the presence of cellular communication between the implant interface and bone, clearly conflicting with the viewpoint that only a dissolution-reprecipitation mechanism results in the formation of the surface apatite layer.[40,52]

Electron diffraction has confirmed the type of precipitate that forms in this interfacial layer to be wholly, or a combination of, HA and OCP.[67-70] Xin et al. have identified a crystallographic

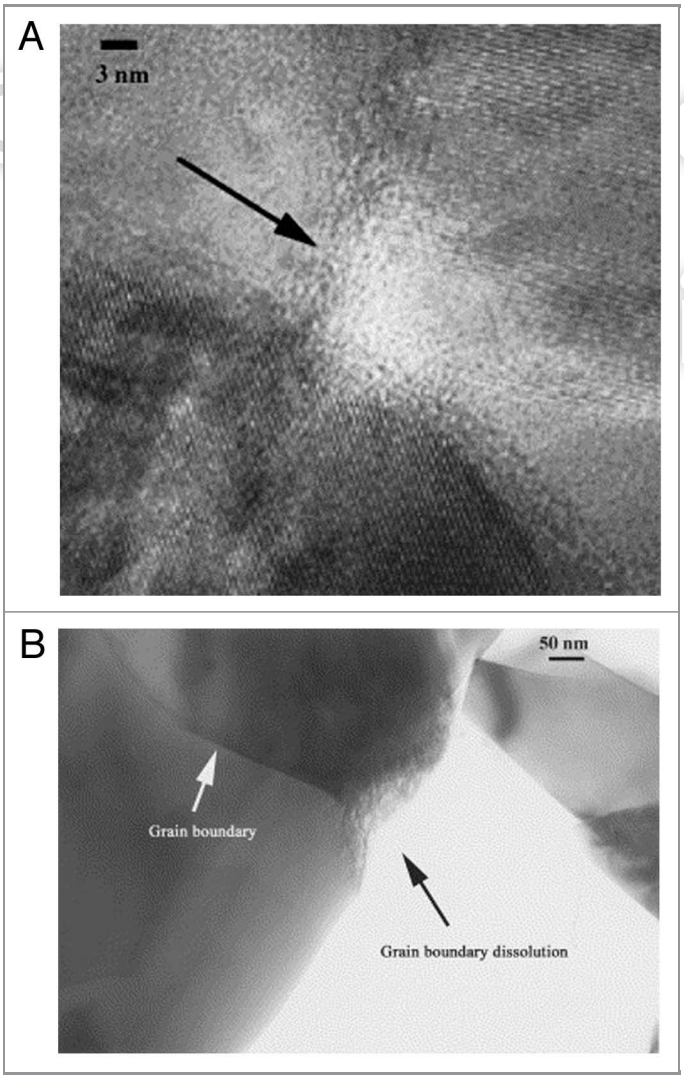

Figure 3. (A) EFTEM micrograph showing the reduced crystallinity at a triple-point junction in Si-substituted HA, (B) TEM micrograph exhibiting increased dissolution at a grain boundary. Reproduced with permission from reference 58.

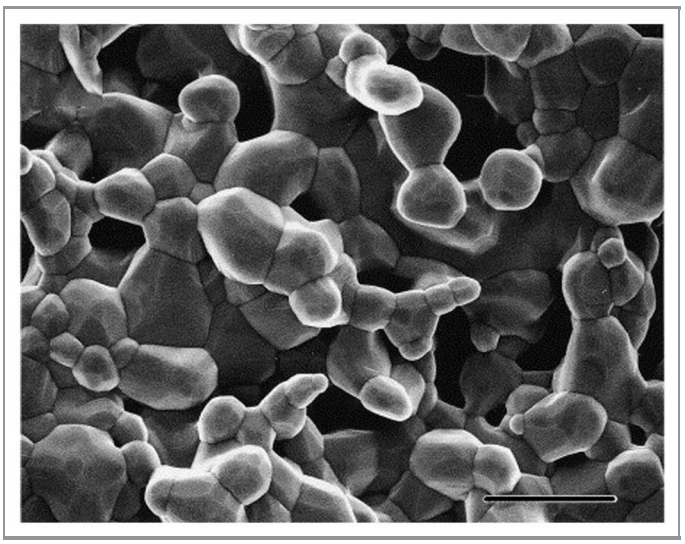

Figure 4. SEM micrograph of HA showing micropores and grain boundaries that contribute to increased dissolution and biocompatibility. Scale bar is 5 μm. Reproduced with permission from reference 71.

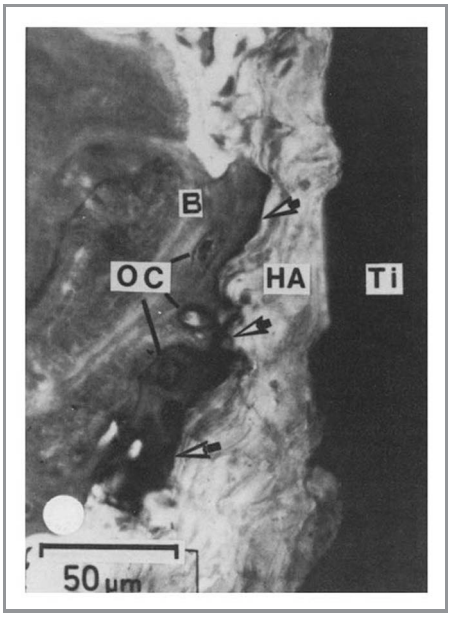

Figure 5. Light micrograph of the HA coated titanium to bone interface. Arrows indicate the dark interfacial region of organic, non-collagenous material. Reproduced with permission from reference 45.

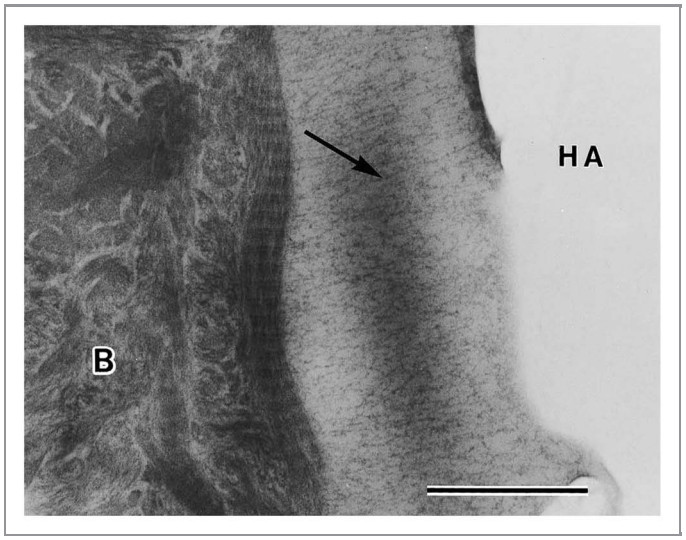

Figure 6. TEM micrograph of an HA particle after 4 weeks in rats. Precipitates are forming perpendicular to the crystal surface indicated by an arrow. Scale bar is 0.5 µm. Reproduced with permission from reference 71.

relationship common to OCP/HA interfaces suggesting the possibility that an in vivo shift from OCP to HA occurs.[70] In the instances of only HA precipitation, Hemmerle et al. have shown that in vivo HA crystal growth follows the same (110) and (001) orientation of the implanted HA, thereby suggesting that epitaxial growth occurs in vivo.[67] Daculsi et al. have also shown crystal growth perpendicular to the surface of the ceramic and electron diffraction confirmed that the c-axes of newly formed precipitates were in agreement with those of the originally implanted material.[64] Furthermore, Fujita et al. showed a fibrillar structure formed perpendicular to the implant surface (**Fig. 6**).[71]

New developments in TEM techniques have enabled three-dimensional imaging of nanostructures. Through the use of electron tomography, the compilation and reconstruction of many images taken over a large angular range, the HA-bone interface has also shown perpendicular crystals in the interfacial zone (**Fig. 7**), while EDS shows a compositional gradient suggesting their formation by a dissolution-reprecipitation mechanism.[32] However, other investigations suggest that crystal formation is not always perpendicular. In the case of a plasma-sprayed HA

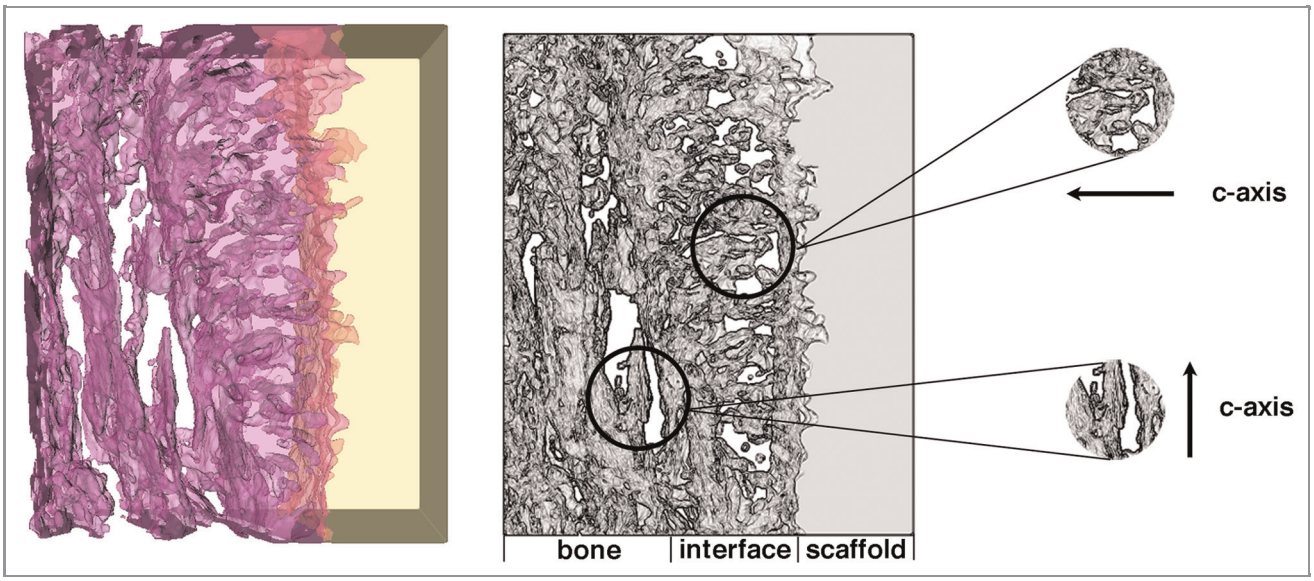

Figure 7. Electron tomograms indicating the orientation of HA crystallites in bone parallel to the HA surface, while crystallites precipitated at the scaffold interface are perpendicular to the surface. Reproduced with permission from reference 72.

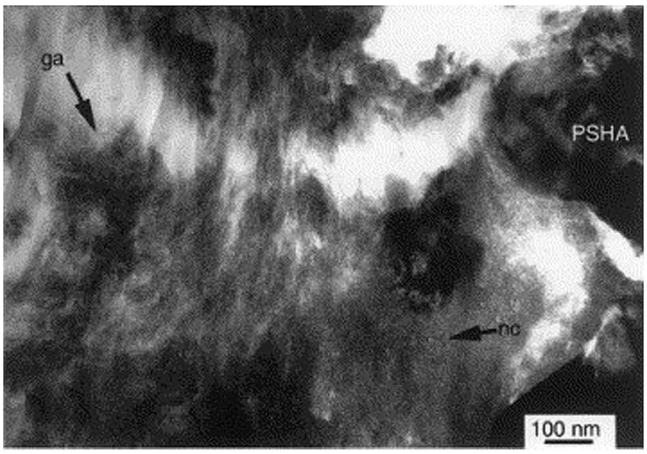

Figure 8. TEM micrograph of bone interfacing to plasma-sprayed HA. A nanocrystalline region is shown (labeled nc) and alignment of HA crystals appears parallel to the PSHA interface. Reproduced with permission from reference 59.

coating, Porter et al. identified a nanocrystalline region and the alignment of HA crystals parallel to the interface after 10-day implantation in dogs (**Fig. 8**).[59]

Light and electron microscopic investigations have provided strong evidence to support the behavior of HA in vivo. While the exact composition and orientation of precipitate formation remains disputed, it is generally accepted that an apatite layer forms on the surface of HA in vivo to facilitate bone growth and attachment.

Conclusions and Future Outlook

Resolving the interface between calcium phosphate materials and bone has been possible through the advancements in microscopy. Techniques such as light, scanning electron, focused ion beam, scanning probe and transmission electron microscopy have all played a role in resolving the bioceramic-bone interface. Indeed, the evolution from light to electron microscopic techniques has enabled a greater understanding of the calcium phosphate to bone interface by providing structural and elemental information on the nanometer or ultrastructural level. While a considerable number of advances have been made in the last decade to improve the ability to resolve the interface between bioceramics and bone, a number of improvements remain to be made. Recent developments enabling three-dimensional interfacial imaging are on the forefront of bioceramic-bone analysis. Our work with Z-contrast electron tomography has demonstrated the usefulness of imaging an interface at both the nanometer scale and in three dimensions, where the resulting tomograms from an HA-bone interface provided a greater amount of information than that obtained purely from two-dimensional projections.[72] Moreover, a paradigm shift in interfacial imaging encourages researchers to aim for non-invasive and non-destructive imaging techniques that provide increased clinical relevance. These aims have led to the increased use of X-ray computed tomography (CT).[60,73,74] In particular, the increased availability of systems such as the NanoSpect/CT has revolutionized the ability to image small-animal bone-implant interfaces in a timely manner.[75] With continual developments in microscopy techniques and capabilities, new discoveries and revelations of the calcium phosphate to bone interface are on the horizon.

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